Morphological and Molecular Diagnosis of Bio-fungus Isolate Penicillium Commune DSKZ and its Laboratory Use in Biological Control of Pathogenic Fungus Sclerotinia Sclerotiorum (Lib) DeBary Causing White Mold on Eggplant

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Abstract

The selected fungus isolate *Penicillium* was diagnosed according to morphological and Molecular characteristics when cultured on PDA culture media. The results of DNA extraction from spp. *Penicillium* fungus that had subjected to a polymerase chain reaction (PCR) showed the possibility of duplicate PCR-amplified products with an expected size of 550 nitrogenous base pairs by polymerase chain reaction (PCR) and in the presence of pair of the Forward primer (ITS1) and Reverse primer (ITS4). This is considered the first recording for this isolate in Iraq, and it was registered in the gene bank by a name of DSKZ, and its code in the gene bank was MT065753.1, which was registered in the National Center for Biotechnology Information (NCBI). Some laboratory tests were conducted on this bio-fungus, where the treatment of antagonism between *P.commune* fungus and the pathogenic fungus *S.sclerotiorum*. gave the highest percentage of inhibition, which amounted to 86.1, with highly significant differences compared to the control treatment, which amounted to 0.00%. The concentrations of 50% and 60% in the non-heat-treated fungal extracts (non-sterile) in terms of colony diameter and the percentage of inhibition for pathogenic fungus have excelled on the rest of the concentrations, which gave the highest values amounted to 0.00, 100%, respectively, for both concentrations. As for the treatment of the thermally treated fungus extracts (sterilized with an autoclave device at a temperature of 121 °C and a pressure of 1.5 kg.cm^{-1} for 20 min), the 60% concentration has excelled on the rest of the concentrations by giving it the highest values in terms of colony diameter and the percentage of inhibition, which amounted to 1.012 cm and 88.8 %, respectively, compared to the control treatment in which the colony diameter and percentage of inhibition amounted to 9.00 cm and 0.00%, respectively. *P.commune* extracts concentrations also caused an increase in the percentage of germination for eggplant seeds at a probability level of 0.05.

Key Words: Penicillium Commune, DSKZ, Sclerotinia Sclerotiorum, White Mold, Eggplant.

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Introduction

The *Penicillium* fungus has shown promising activity in microbial biodegradation research in relation to environmental pollutants, where a study in 2014 determined the ability of this species to biodegrade industrial oil residues (Sailaja et al, 2016). Although the bio-oil removal rate was dependent on oil volume, pH level, and incubation period, however, The optimum conditions led to removal rate amounted to 95.4% of waste oil by *P.commune*.

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The fungus may be a new source in industrial

applications regarding biodegradation of oil residues in the environment using biological means (Esmaeili and Sadeghi, 2014) although *P. commune* has no known activity of penicillin. Environmental isolation of the fungus has been shown to produce statin, simvastatin, mevastatin, and anti-disease products. The fungi species were able to reduce the growth of two pathogenic bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by affecting biofilms in a laboratory environment. In addition, there was evidence of lovastatin production from the environment in which the *P.commune* fungus is present besides its ability to improve the performance of antibiotics oxacillin, this species has proven to be a new and promising source in the production of anti-disease products for medical applications (Diblasi et al., 2015). Where this type of fungi uses statins as a defense mechanism against other microorganisms that require sterols and or isoprenoids derived from mevalonate for their growth, statins inhibit HMG-CoA reductase, an enzyme that plays a major role in cholesterol production (Manzoni and Rollini, 2002). In addition, this type of fungus may stimulate and encourage germination, this was confirmed by (Marjan, 2006), where it was found that yellow corn seeds were stimulated to germination when treated with the spore suspension for types of fungus *Penicillium*. This may be attributed to the nature of the secretions secreted by these types of fungal. The first recording for this isolate, which was named by DSKZ and with number: MT065753.1 in the gene bank, and Because of the importance of this bio-fungus *p.commune* and its high efficacy and its secretion of many antibiotics, Therefore, this study aimed to know the antagonistic potential for the isolate of this bio-fungus against plant diseases, especially against the pathogenic fungus *S.sclerotiorum*. Which results due to infection, which causes great economic damage due to its wide family plant range, its resistance to inappropriate conditions, and its large spread in the Iraqi environment.

Materials and Methods

Morphological and Molecular diagnosis of the P.commune fungus

The fungus isolate *p.commune* was activated after growing the fungus by the single spore method in Petri dishes with a diameter of 9 cm containing PDA culture media, which was obtained from the College of Science / University of Babylon. The dishes were incubated at a temperature of 25°C, and after 5 days, the fungus was diagnosed based on the traits of the fungal culture, the nature of the mycelium and the spores it forms, using the approved taxonomic keys. The molecular diagnosis of the fungus was then conducted using the molecular diagnosis method for this isolate (PCR) to confirm the morphological diagnosis. (Shihab, and Abood, 2019).

Molecular diagnosis for the bio-fungus p.commune using polymerase chain reaction (PCR) technology. It includes several steps:

1- Extraction of deoxyribonucleic acid (DNA) from the isolate of the fungus p.commune. isolated in this study

DNA was extracted from isolates of the fungus *p.commune* using the kit (Cat. No: FAPGK100) supplied by Favorgen, Taiwan, China.

2- Estimating the concentration and purity of the DNA extract

The DNA concentration was estimated using a spectrophotometer under a wavelength of 260nm, and the DNA concentration was determined by the following equation:

DNA concentration (µg / ml) = value of optical absorption at a wavelength of 260nm × 50 × dilution factor.

The purity of DNA was also determined by applying the following equation described by:

$$\text{DNA purity} = \frac{\text{The amount of absorption at a wavelength of 260 nm}}{\text{The amount of absorption at a wavelength of 280 nm}}$$

The DNA extracted from the isolate of the *P.commune* fungus at -20°C was then preserved until tested using PCR technology.

3- Using polymerase chain reaction (PCR) technology

For the purpose of diagnosing the fungi isolated in this study, a polymerase chain reaction (PCR) test was conducted using the Maxime PCR PreMix (i-Taq), (Cat. No. 25026) kit supplied by the Korean iNtRoN company. The polymerase chain reaction was conducted with a total volume of 20 µl containing 1 µl of each Forward primer (TCCGTA GGTGAACCTGCGG:ITS1) and Reverse primer (TCTCTCCGCTTATTGATATGC:TS4) and 1 µL of the extracted DNA. All of the above ingredients in the tube provided by the manufacturer and the volume

was completed with Nuclease-free water to 20 µl. The DNA for the fungus isolate *P.commune* was replicated using steps and conditions of polymerase chain reaction (PCR) (Zhang et al., 2012).

4- Electrophoresis using agarose gel electrophoresis

A layer of agarose gel was prepared after taking 1 g weight of agarose powder and dissolving it in 100 ml of buffer solution (Tris boric acid EDTA buffer × 1TBE). 5 µl of ethidium bromide pigment was added after the solution temperature was reduced to 45-40°C. The dissolved Agarose containing ethidium bromide pigment was poured and left to solidify at room temperature. When the agarose layer solidifying, The comb was carefully lifted and the mold was returned to its place in the electrophoresis device. 1×TBE solution was then added to the electrophoresis tank, covering the agarose layer with a height of approximately 1 cm. 10 µl of DNA was added by polymerase chain reaction (PCR) to each well of the previously prepared agarose gel layer. 5 µl of DNA ladder were added to the hole on the left side of the added samples. The electrodes of the power supply were connected to the electrical current and it worked at 50 mA for a period of one hour. After completing the transfer of samples, the agarose gel layer containing the DNA bands (PCR products) was examined under UV rays (UV transillumination) and photographs were taken.

5- DNA sequencing analysis of the fungus isolate p.commune

For the purpose of diagnosing the isolated fungi, PCR amplicons were sent from isolate *P.commune* by polymerase chain reaction (PCR) with primers (ITS1) and (ITS4) to the Korean company Macrogen.

The antagonistic potential of the bio-control fungus p.commune in inhibiting the isolation of the pathogenic fungus S.sclerotiorum

The experiment was conducted to study the antagonistic relationship between the pathogen *S.sclerotiorum* and the *P.commune* fungus obtained using the dual culture method between the isolated fungi and the bio-control fungus on P.D.A. In Petri dishes with a diameter of 9 cm, the edge of the first half of the dish was inoculated with a disk of 0.5 cm diameter of the fungal growth for the colony of pathogenic fungus *S.sclerotiorum*, and the edge of the other half was inoculated with a disk similar to the bio-control fungus *P.commune*. The experiment was conducted with a rate of four replicates per treatment, and 4 dishes were left as the control treatment, meaning pathogenic fungus is alone. The dishes were placed in the incubator at a temperature of 25°C. After the growth of pathogenic fungi reached the edge of the dish, the antagonism was estimated according to measuring the diameters of the growth rate of the pathogenic fungi by taking the average of two perpendicular diameters from the back of the dish passing through the center of the colony represented by the disc. The average of antagonism was estimated according to the scale of (Bell et al., 1982), which consisted of 5 degrees as follows:

Grade 1- Antagonistic fungus cover the entire dish area.
Grade 2 - Antagonistic fungus cover two-thirds of the dish area.
Grade 3- Antagonistic fungus and pathogenic fungus each cover half of the dish.
Grade 4- Antagonistic fungus cover one-third of the dish area and pathogenic fungi cover two-thirds of the dish area.
Grade 5- The pathogenic fungus covers the entire area of the dish.

The biological agent is considered antagonistically effective when it shows a degree of antagonism of 2 or less with the isolate of the pathogenic fungi under study, the percentage of inhibition for fungal growth was then calculated according to the equation of (Montealegre et al., 2003).

$$\text{The percentage of inhibition} = \frac{\text{The average diameter of the control colony} - \text{the average diameter of the treated colony}}{\text{The average diameter of the control colony}} \times 100$$

Preparing fungus Extract p.commune

Potato Dextrose Broth (PDB), and kept in the refrigerator at a temperature of (5°C) until use. This filter was used to know its effect on the national growth of the pathogenic fungus, as well as its effect on the percentage of germination of eggplant seeds.

Studying the effect of Extracts of the untreated and

heat-treated bio-control fungus p.commune on the growth of the pathogenic fungus S.sclerotiorum on PDA culture media:

The Poisoned food method was used to estimate the effectiveness of Extracts of the untreated and heat-treated fungus *p.commune* against the pathogenic fungus *S.sclerotiorum* to know the effect of enzymes and mycotoxins for this bio-fungus after the fungal extracts was obtained free of reproductive units. The Extracts was divided into two parts, where the first part was the previously prepared Extracts of the fungus *p.commune* was added to the PDA media after sterilization with an autoclave device at a temperature of 121 °C and a pressure of 1.5 kg.cm-2 for 20 min and before the solidification stage at concentrations of (20, 30, 40, 50 60%) and individually, taking into account the adjustment of the percentage of agar added to the culture media before sterilization. The media containing antagonistic fungus extracts were poured into sterile Petri dishes (with a diameter of 9 cm) and left to solidify. Each dilution treatment was repeated four times with the implementation of the control treatment using culture media not treated with Extracts as a control treatment. As for the second section of the fungal Extracts, the same concentrations mentioned above were added before the media was placed in the sterilizer, where it was sterilized with the fungal media, after extracting the media from the sterilizer and before the stage of solidification, the sterile extracts mixed with the culture media were poured into sterile Petri dishes (with a diameter of 9 cm). It was left to cool, the media containing the non-sterile and thermally sterile Extracts were then inoculated with discs each with a diameter of 0.5 cm from the colony of the pathogenic fungus *S.sclerotiorum* and at the age of 5 days in the center of each dish for the concentrations of the experiment, The dishes were incubated in the incubator at a temperature of 25 °C, and when the growth of pathogenic fungi in the control treatment reached the edge of the dish, the diameters of the growth rate of pathogenic fungi were measured by taking the average of two perpendicular diameters from the back of the dish passing through the center of the colony represented by the disc, and the percentage of inhibition was calculated according to the equation of (Montealegre et al., 2003).

Effect of fungus Extracts p.commune on the percentage of germination for eggplant seeds in dishes and on PDA media with different concentrations

Several different concentrations were prepared from the extracts of the fungus *p. commune* in PDA culture media. The Extracts concentrations (20, 30, 40, 50, 60%) were prepared in Petri dishes as in the previous paragraph. They were poured into Petri dishes with a rate of (4 replications/ concentrations). After that, a quantity of eggplant seeds was superficially sterilized by sodium hypochlorite at a concentration of 2% for two minutes, it was then washed with sterile distilled water and dried on filter paper. Eggplant seeds were cultured on extracts concentrations with a rate of 25 seeds per dish with the implementation of the control treatment using eggplant seeds on the PDA media without any addition, the percentage of germination after 10 days was then calculated according to the equation of (Al-Rawji, 2005).

$$\text{The percentage of germination} = \frac{\text{number of germinated seeds}}{\text{total cultured seeds}} \times 100$$

Results and Discussion

Morphological diagnosis

The selected isolate *Penicillium* was diagnosed according to the Morphological and microscopic characteristics when culture on PDA media. where the isolates gave spherical conidia spores carried in irregular chains on the Conidiophores, the branches of Conidiophores be either singly or in groups and in bundles. The color of the conidia is green and the colony is velvety with white edges from the top. The bottom surface of the colonies was light yellow or pale yellow. The above specifications apply to the type of fungus *p.commune* according to the taxonomic keys mentioned by (Pitt et al., 1986; Samson et al., 2001) as shown in Figure (1), which shows a picture of the bio-fungus on PDA culture media.

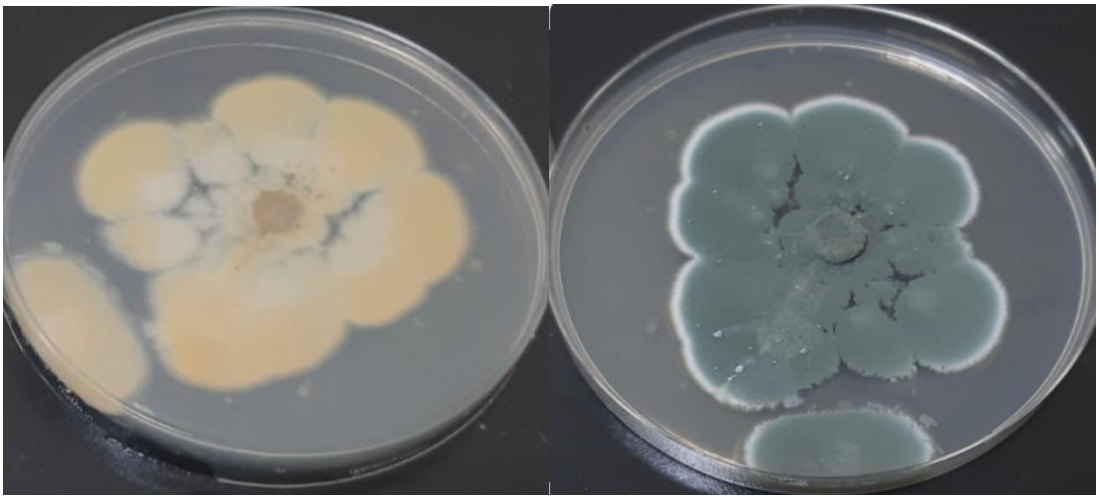

Figure 1. The shape and color of the colony for the fungus isolate *p.commune* on the center of the PDA, the first image on the right represents the upper surface of the colony and the second image on the left represents the lower surface of the colony (the back of the dish)

Molecular diagnosis for the fungus isolate p.commune

The results of DNA extraction from *Penicillium* spp. fungus that had subjected to a polymerase chain reaction (PCR) showed the possibility of duplicate PCR-amplified products with an expected size of 550 nitrogenous base pairs by polymerase chain reaction (PCR) and in the presence of pair of the Forward primer (ITS1) and Reverse primer (ITS4). The results of the nucleotide sequence analysis of the multiplexed DNA products from the fungal isolate using the BLAST program to compare with the data available at the US National Center for Biotechnology Information (NCBI) proved that this isolate belongs to the fungus *p.commune* as shown in Figure (2). It was also shown by comparing the sequences of nitrogenous bases of the isolates diagnosed in this study that the isolates diagnosed in this study gave similarity rates of 99% with the mentioned isolates as shown in Figure (3). It is noted from the results of this study that the isolate of *p.commune* was genetically different from other isolates diagnosed and registered in the National Center for Biotechnology Information (NCBI) as shown in Figure (4). Therefore, it was registered in the mentioned center and under the entry number is MT065753.1, and this is the first record of this isolate in Iraq and it was registered in the gene bank in the name of DSKZ.

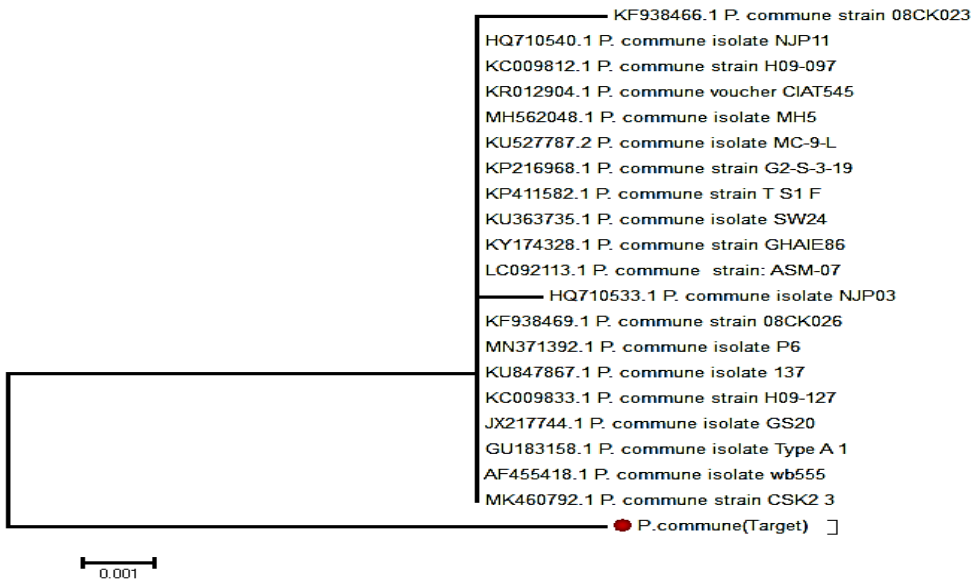

Figure 2. Neighbor-Joining tree showing the genetic relationship for *P.commune* isolate in this study and other isolates of the same fungus previously registered in the National Center for Biotechnology Information (NCBI)

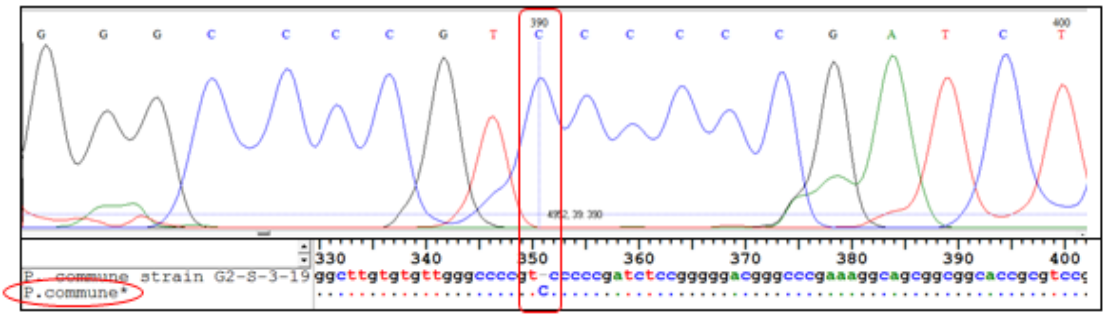

Figure 3: The difference in the sequence of some nitrogenous bases for the PCR product that duplicates from the *P.commune* isolates isolated in this study and the most closely related P. commune isolates that registered in the National Center for Biotechnology Information (NCBI). * The *P.commune* fungus isolated in this study

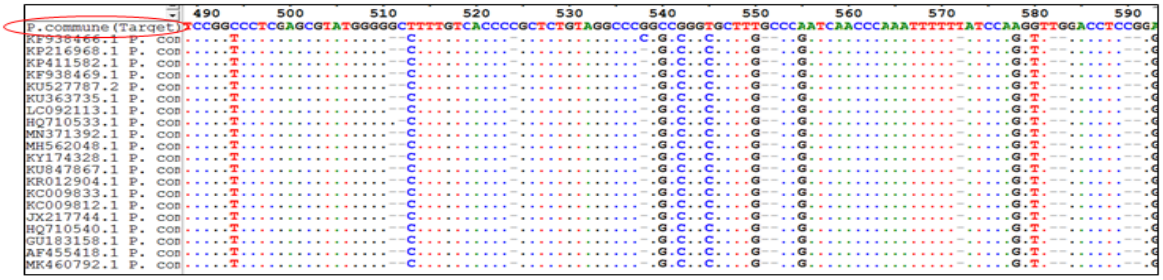

Figure 4. Similarities and differences in some regions of the sequence alignments for the PCR-amplified products of the *P.commune* isolate, as well as other isolates of the same fungus, previously registered in the National Center for Biotechnology Information (NCBI). Similar nitrogenous bases are represented by dots.

Testing the antagonistic potential of P.commune against S.sclerotiorum using the dual culture method in vitro conditions

Table (1) shows the results of this test the presence of a high antagonistic potential between the bio-control element *P. communie* and the pathogenic fungus *S. sclrerotiorum* in addition to its effect on the growth of pathogenic fungi in vitro. The results of the fungus used in bio-control achieved a high antagonistic potential amounted to (2) according to the scale set by (Bell et al., 1982), which is the second degree of this scale that set by this scientist, and after four days of culturing the pathogenic fungus, until the pathogenic fungus access the edge of the dish, where it gave The treatment of P. commune with the pathogenic fungus gave a high percentage of inhibition which amounted to (86.1%) as shown in Figure (5) and the Sclerotia of the pathogenic fungus amounted to 55.9% compared to the control treatment which amounted to 0.0%. This is due to the production of antibiotics by this type of fungi, which is one of the important mechanisms of the fungus in bio-control. There is evidence that lovastatin is produced from the environment in which the *P.commune* fungus is present, besides its ability to improve the performance of antibiotics oxacillin. The environmental isolate of the fungus has been shown to produce statins and anti-disease products and this species has proven to be a new and promising source in the production of anti-disease products for medical applications (Diblasi et al., 2015). This result agrees with (Wajda Al-Jubouri, 2013), who found that the *Penicillium* sp. fungus has recorded a degree of antagonism amounted to (2) according to the (Bell Scale, 1982) against the pathogenic fungus *S.sclrerotiorum*.

Table 1. The diameter of the pathogenic fungus colony with the percentage of inhibition for the pathogenic fungus by the bio-fungus *P.commune*

No.	Treatment	The diameter of colony	The percentage of inhibition
1	*P. commune* + Ss	**1.250	**86.1
2	*P. commune*+ Sclerotia	2.77	55.9
3	fungus Ss alone (control)	9.00	0.0
4	L.S.D. (5%)	0.856	31.74

Sc* represents the *Sclerotinia sclerotiorum* fungus
**Each number in the table represents an average of four replicates

Figure 5. From the right side, an image showing the biological antagonism of *p.commune* with the pathogenic fungus, and from the left side the pathogenic fungus alone (control)

Studying the effect of Extracts of the untreated and heat-treated bio-control fungus p.commune on the growth of the pathogenic fungus S.sclerotiorum on PDA culture media

This experiment was conducted to investigate the effect of enzymes and toxins of bio-fungus *p.commune*, where the results of table (2) showed the effect of several concentrations of the fungus Extracts *p.commune* on reducing the growth of the pathogenic fungus *S.sclerotiorum* with different percentages of inhibition and according to the type of Extracts that is neither heat-treated nor heat-treated (sterile or non-sterile) on PDA culture media. The results indicate that there are highly significant differences in the colony diameter and the percentage of pathogenic fungus inhibition by concentrations of antagonistic fungus extracts and between the fungus treatment alone (fungus without Extracts), where the two concentrations 50% and 60% in the non-sterile extracts, respectively, exceeded the rest of the concentrations, which gave the highest values in terms of colony diameter and percentage of inhibition, which amounted to 0.00 and 100%, respectively, for both treatments. The concentration treatment (20%) for unheated (non-sterile) filters gave a diameter growth rate of the pathogenic fungus *S.sclerotiorum*, which amounted to 2.6 cm and a percentage of inhibition less than the rest of the concentrations amounted to 71.12%, compared to the control treatment, where the diameter of the colony and the percentage of inhibition was 9.00 cm 0.00 %, respectively. This may be due to the fact that the extracts of this fungus contains many substances that inhibit the growth of the isolate for the pathogenic fungus. These results agree with (Al-Jubouri, 2013), who found that *Penicillium* sp. extracts recorded 100% percentage of inhibition for *S.sclerotiorum* at 60% concentration, which also found that *Penicillium* sp. extract contains alkaloids, tannins, carbohydrates, saponins, and glycosides. We conclude from this experiment that the higher the extracts concentration, the greater the inhibitory effect on the growth of the pathogenic fungus *S.sclerotiorum* as shown in Figure (6). The results of the antagonistic effect of the fungus Extracts may be attributed to its production of antibiotics, which is one of the mechanisms of the fungus in bio-control, where the Extracts of the fungus *Penicillium* sp. contains many metabolites such as Griseofulvin and Penitrem (Ali et al., 2011). Table (2) shows the effect of heat-treated fungus extracts (sterilized with an autoclave device) at a temperature of 121 °C and a pressure of 1.5 kg/cm for 20 min, on the colony diameter and the percentage of inhibition for the pathogenic fungus *S.sclerotiorum*, where the 60% concentration exceeded the rest of the concentrations, which gave the highest values in terms of the colony diameter and the percentage of inhibition which amounted to (1.012 cm and 88.8%), respectively, compared to the control treatment in which the diameter of the colony and the percentage of inhibition amounted to (9.00 cm and 0.00)%, respectively, followed by the treatment of concentration 50 and 40%, in

which the diameter of the colony was 1.700 and 1.775 cm, respectively, with a percentage of inhibition amounted to 81.1 and 80.3, respectively, and there were no significant differences between them and the 60% concentration treatment for the heat treated filter. The 20% concentration treatment for the heat-treated filters gave an average growth diameter and a lower percentage of inhibition than the rest of the concentrations, which amounted to 2,800 cm and 68.9%, respectively, compared to the control treatment in which the diameter of the colony and the percentage of inhibition amounted to 9.00 cm and 0.00%, respectively, as shown in Figure (7). The results of the heat-treated antifungal extracts are also attributed to the fact that it contains mycotoxins that inhibit the pathogenic fungus *S.sclerotiorum*. These results agree with (Nicoletti and Stefano, 2012), where it was found that the Extracts of the fungus *Penicillium restrictum* contains some toxins such as gliotoxin, which is a toxin. Fungi do not have antibiotic properties, but have the most important role in antagonism fungi, some types of this antagonistic fungus produce secondary metabolites such as Dihydrocurvularin, Pyran, Triene, and Restricticin, which are characterized by their wide activity against a wide range of yeasts and filamentous fungi. These results also agree with (Dhliwayo, 2008) where it was found that *P.citrinum* is one of the mycotoxin-producing fungi that inhibits the growth of the mycelium of Sclerotinia species. Also, adding 20% of the fungus filter *P.citrinum* to the growth media of *S. minor* and *S.rolfsii* completely inhibited the growth of these two pathogenic fungi.

Table 2. Effect of different concentrations of Extracts of the fungus *p.commune* on the growth of the isolate of pathogen fungus *S.sclerotiorum* on PDA culture media

No.	Concentration of Extracts % untreated	Colony diameter (cm)	percentage of inhibition (%) unheated treatment	Diameter of Colony (cm) for hot Extracts	percentage of inhibition (%) for hot Extracts
1	20	2.600	71.12	2.800	68.9
2	30	2.125	76.38	2.400	57.6
4	40	0.750	91.67	1.775	80.3
5	50	0.000	100.00	1.700	81.1
6	60	0.000	100.00	1.012	88.8
7	Fungus S.s alone (control)	9.000	0.00	9.000	0.0
8	L.S.D. (5%)	0.4987	4.982	0.5962	21.45
1	20	2.600	71.12	2.800	68.9

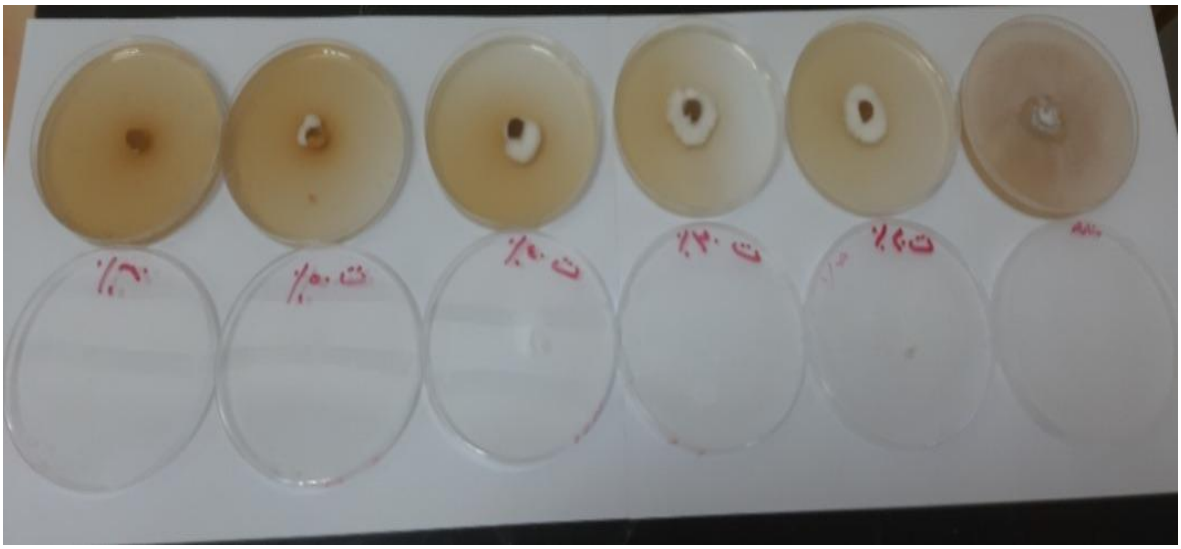

Figure 6. Shows the antagonism of the Extracts of the fungus *p.commune* with pathogenic fungi at different concentrations with control treatment

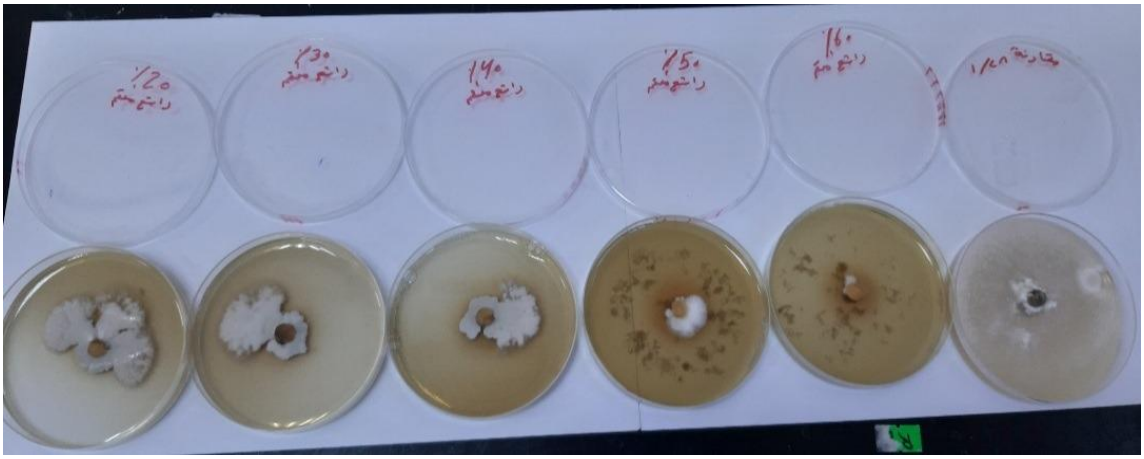

Figure 7. Shows the antagonism of the heat-treated Extracts of the fungus *p.commune* with pathogenic fungus at different concentrations with control treatment

Effect of concentrations of fungus Extractss p.commune on eggplant seed germination in Petri dishes on PDA media

Table (3) indicates that the extracts of the fungus *p.commune* caused an increase in the germination of eggplant seeds at a probability level of 0.05 and with highly significant differences compared to the control treatment, where the concentration of 50 and 60% gave the highest results in germination, where the percentage of germination amounted to 100%, While the percentage of seed germination in the control treatment was 83.00%, followed by the concentration treatment 40 and 30%, where the percentage of germination amounted to 99 and 98%, respectively, while the treatment of Extracts 20 gave the lowest percentage of germination, amounted to 96% with a significant difference from the rest of the concentrations of fungal extracts. These results are attributed to the absence of negative effects for the fungal extracts of *P.commune*. It may also be due to the secretion of this type of fungus to growth regulators that encourage the germination and growth of seeds. these results agree with (Al-Jubouri, 2017) who showed the effect of several concentrations of the fungus *P.corylophilum* Extracts on the percentage of germination for radish seeds. The fungus extracts *P.corylophilum* had a significant effect on the percentage of germination for radish seeds, where the concentration 50% gave the highest percentage of germination for the seeds which amounted to 100%, while the concentration of 10 and 25% gave a percentage of germination amounted to (88 and 92%), respectively, compared to the control treatment, which amounted to (84)%.

Table 3. Represents the effect of *p.commune* extracts concentrations on the germination of eggplant seeds in Petri dishes on the PDA media.

No.	Concentration of Extracts %	The number of germinated seeds	percentage of germination
1	20	24.00	96.00
2	30	24.50	98.00
4	40	24.75	99.00
5	50	25.00	100.00
6	60	25.00	100.00
7	Control (seed without extracts)	20.75	83.00
9	L.S.D. (5%)	0.818	3.571
1	20	24.00	96.00
2	30	24.50	98.00

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